FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	
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U.S. APPLICATION NO (IF KNOWN, SEE 37 CFR	
09/926681	
INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE
PCT/PT00/00005	31 MAY 2000
PRIORITY DATE CLAIMED	
31 MAY 1999	

TITLE OF INVENTION

CULTURE MEDIUM FOR DETECTION OF DEKKERA AND BRETTANOMYCES

APPLICANT(S) FOR DO/EO/US

Virgilio B. LOUREIRO, et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) The submission must include items (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S C. 1 821 - 1 825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4)
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☐ Certificate of Mailing by Express Mail
23. ☒ Other items or information.

Request for Consideration of Documents in International Search Report
Notice of Priority / PCT/IB/304 / PCT/IB/308

JC13 Rec'd PCT/PTO 30 NOV 2001

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
09/926681	PCT/PT00/00005	211341US0XPCT

24. The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :		
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**		
☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**		
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$890.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$130.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	- 20 =	0	x $18.00	**$0.00**	
Independent claims	- 3 =	0	x $84.00	**$0.00**	
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**	
TOTAL OF ABOVE CALCULATIONS =				**$1,020.00**	
☐ Applicant claims small entity status. See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				**$0.00**	
SUBTOTAL =				**$1,020.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**	
TOTAL NATIONAL FEE =				**$1,020.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐				**$0.00**	
TOTAL FEES ENCLOSED =				**$1,020.00**	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of **$1,020.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **15-0030** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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REGISTRATION NUMBER

Nov. 30 2001
DATE

211341US-0X PCT

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF: :

VIRGILIO B. LOUREIRO ET AL. :

SERIAL NO: 09/926,681 : ATTN: APPLICATION BRANCH

FILED: NOVEMBER 30, 2001 :

FOR: CULTURE MEDIUM FOR DETECTION OF DEKKERA AND BRETTANOMYCES

PRELIMINARY AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

SIR:

Prior to examination on the merits, please amend the above-identified application as follows.

IN THE CLAIMS

Please amend the claims as shown in the marked-up copy following this amendment to read as follows.

1. (Amended) A differential culture medium for the enumeration of food and beverage contaminant yeasts of the *Dekkera* and *Brettanomyces* genera, characterized in that it comprises a nutrient base, ethanol as the only energy source, ρ-cumaric acid, an acid-base indicator with turning points in the acid range, an inhibitor antibiotic for sensitive yeasts species, and optionally a bacteria growth inhibitor and agar-agar.

9. (Amended) A culture medium according to claim 1, characterized in that it contains all the components except agar-agar, to detect and identify yeasts of the *Dekkera*

and *Brettanomyces* genera in food and beverage products containing mixed populations of yeasts, bacteria and particularly filamentous fungi.

11. (Amended) A differential culture medium for enumeration of food and beverage contaminant yeasts of the *Dekkera* and *Brettanomyces* genera according to claim 1, characterized by the sterilization of all the components is done by filtration, except for the agar-agar which is sterilized in autoclave; the addition under aseptic conditions to this solution, after agar-agar cooling and before it solidifies, of all the other components of the medium, previously sterilized by filtration; and the dispensing of the medium into Petri dishes so that it solidifies.

12. (Amended) A process for the detection and/or identification of yeasts of the *Dekkera* and *Brettanomyces* genera, characterized by the use of a differential culture medium for enumeration of food and beverage contaminant yeasts of the *Dekkera* and *Brettanomyces* genera, comprising a nutrient base, ethanol as the only energy source, ρ-cumaric acid, an acid-base indicator with turning points in the acid range, an inhibitor antibiotic for some of the yeast species, and optionally a bacteria growth inhibitor and agar-agar.

14. (Amended) A process for the detection and/or identification of yeasts of *Dekkera* and *Brettanomyces* genera according to claim 12, characterized in that it is applied to the detection and enumeration of yeasts of the *Dekkera* and *Brettanomyces* genera in food and beverage products samples.

15. (Amended) Use of a culture medium according to claim 1, for inclusion in an identification gallery, together with other yeast identification tests.

16. (Amended) Use of a culture medium according to claim 1 in an industry, particularly in the quality and process control in a food and beverage industry.

17. (New) Use of a culture medium according to claim 10, for inclusion in an identification gallery, together with other yeast identification tests.

18. (New) Use of a culture medium according to claim 11, for inclusion in an identification gallery, together with other yeast identification tests.

19. (New) Use of a culture medium according to claim 10 in an industry, particularly in the quality and process control in a food and beverage industry.

20. (New) Use of a culture medium according to claim 11 in an industry, particularly in the quality and process control in a food and beverage industry.

REMARKS

Claims 1-20 are active in the present application. Claims 1 and 12 have been amended to limit ethanol as the only energy source. Claims 9, 11 and 14-16 have been amended to remove multiple dependencies. Claims 17-20 are new claims. Support for the new claims is found in the original claims. Support for the amendment is found on sheet 6A of the Article 34 Amendment. No new matter is believed to have been added by this amendment. An action on the merits and allowance of claims is solicited.

Respectfully submitted,

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Marked-Up Copy
Serial No:

Amendment Filed on:
3-14-2002

IN THE CLAIMS

Please amend the claims as follows.

--1. (Amended) A differential culture medium for the enumeration of food and beverage contaminant yeasts of the *Dekkera* and *Brettanomyces* genera, characterized in that it comprises a nutrient base, ethanol as the only energy source, p-cumaric acid, an acid-base indicator with turning points in the acid range, an inhibitor antibiotic for sensitive yeasts species, and optionally a bacteria growth inhibitor and agar-agar.

9. (Amended) A culture medium according to [any one of the preceding claims] claim 1, characterized in that it contains all the components except agar-agar, to detect and identify yeasts of the *Dekkera* and *Brettanomyces* genera in food and beverage products containing mixed populations of yeasts, bacteria and particularly filamentous fungi.

11. (Amended) A differential culture medium for enumeration of food and beverage contaminant yeasts of the *Dekkera* and *Brettanomyces* genera according to [the preceding claims] claim 1, characterized by the sterilization of all the components is done by filtration, except for the agar-agar which is sterilized in autoclave; the addition under aseptic conditions to this solution, after agar-agar cooling and before it solidifies, of all the other components of the medium, previously sterilized by filtration; and the dispensing of the medium into Petri dishes so that it solidifies.

12. (Amended) A process for the detection and/or identification of yeasts of the *Dekkera* and *Brettanomyces* genera, characterized by the use of a differential culture medium for enumeration of food and beverage contaminant yeasts of the *Dekkera* and *Brettanomyces* genera, comprising a nutrient base, [a non-fermentable energy source] ethanol as the only energy source, ρ-cumaric acid, an acid-base indicator with turning points in the acid range, an inhibitor antibiotic for some of the yeast species, and optionally a bacteria growth inhibitor and agar-agar.

14. (Amended) A process for the detection and/or identification of yeasts of *Dekkera* and *Brettanomyces* genera according to [claims 12 and 13] claim 12, characterized in that it is applied to the detection and enumeration of yeasts of the *Dekkera* and *Brettanomyces* genera in food and beverage products samples.

15. (Amended) use of a culture medium according to [claims 1 and 11] claim 1, for inclusion in an identification gallery, together with other yeast identification tests.

16. (Amended) Use of a culture medium according to [claims 1 to 11] claim 1 in an industry, particularly in the quality and process control in a food and beverage industry.

Claims 17-20 (New).--

CULTURE MEDIUM FOR DETECTION OF *DEKKERA* AND *BRETTANOMYCES*

DESCRIPTION

Object of the Invention

The present invention refers to a culture medium for the differential detection and enumeration of food and beverage contaminant yeasts of the *Dekkera* and *Brettanomyces* genera, containing ethanol and *p*-cumaric acid in its preparation. It is also an object of the present invention a method using said culture medium for the differential detection and enumeration of yeasts of the *Dekkera* and *Brettanomyces* genera. It is still an object of the present invention the use of said culture medium in a yeast identification gallery.

The objective of the present invention consists in providing the food and beverage industry with a culture medium and a method using said culture medium, able to isolate, differentially detect and number yeasts of the *Dekkera* and *Brettanomyces* genera, by means of the color changing of the culture medium and of the colonies therein developed, and production of a characteristic phenol-like aroma.

State of the Art

The study and characterization of the yeast microflora present in many different habitats (*e.g.* beverages, food, natural substracts) generally involves a first strain isolation and purifying stage in generic yeast culture media, which is followed by a second identification stage by classical taxonomic methods or by molecular biology based techniques. The slow development of the yeasts of the *Dekkera* and *Brettanomyces* genera makes its isolation in the commonly used media extremely difficult, since these yeasts are overtaken by faster developing ones that coexist with them. This fact makes their detection and

enumeration, as well as their later identification in food and beverages, rather difficult, this being generally accomplished through the use of very slow, work intensive, and technical skill demanding classical techniques or through molecular biology techniques, involving the use of expensive reactants, molecular probes or primers not always promptly available in the market, and of skilled operators.

It was possible to establish beyond any doubt that these yeasts are involved in the production of a serious organoleptic defect in wines - "horse sweat" - particularly in those that are aged in oak casks. (Chatonnet, *et al.* 1992, *J. Sc. Food Agric.*, 60,165-178). Since then, their detection and enumeration in wines became essential, arising the need for the development of swift methods for that effect. The field bibliography discloses suitable means for the detection and enumeration of yeasts of the *Dekkera* and *Brettanomyces* genera based on this species resistance to cycloheximide and their acidifying ability (Chatonnet, *et al.* 1992, *J. Sc. Food Agric.*, 60,165-178; Fugelsang, K. *et al.*1993. Ed. Barry H. Gump. ACS Symposium series 536, *American Chemical Society*, Washington. Cap. 7, 110-119; Alguacil, M. *et al.* 1998. *Aliment. Equipos Tecnol*, 10, 81-85). However, the disclosed media were not entirely satisfactory, since they were not selective enough to prevent the growing of fast developing species, and also were not totally differential.

Therefore, there is a real and effective need for a culture medium and method for the easy and swift identification of yeasts of the *Dekkera* and *Brettanomyces* genera, namely to provide the food and beverage industry a swift method for the isolation, differential detection and enumeration of yeasts of the *Dekkera* and *Brettanomyces* genera.

Description of the Invention

It was surprisingly found that using a culture medium containing ethanol (1) and *p*-cumaric acid for *Dekkera* and *Brettanomyces* genera yeasts growth, these

(1) as the only energy source

produce 4-ethylphenol and acetic acid in a characteristic and exclusive form, compared to other yeasts, after incubation for 5 to 12 days. Furthermore, other species of yeasts, usually coexistent with the yeasts of the *Dekkera* and *Brettanomyces* genera, which much faster development disallow their detection, are selectively inhibited.

According to the invention, a culture medium was developed, which is partially selective and totally differential for yeasts of the *Dekkera* and *Brettanomyces* genera, which is based on 4-ethylphenol detection, by its characteristic aroma, and acetic acid, by means of an adequate acid-base color change, produced by those yeasts when developed in a ethanol and *p*-cumaric acid containing medium.

Therefore, the present invention refers to a culture medium for the differential detection and enumeration of food and beverage contaminant yeasts of the *Dekkera* and *Brettanomyces* genera. Said medium comprises a nutrient base, ethanol as a non-fermentable energy source and inhibiting other yeasts species, *p*-cumaric acid as an aromatic compound promoting substract produced by said yeasts species, an acid-base indicator whose turning points lie within the acid range (particularly bromocresol green), an antibiotic that inhibits several species of yeasts (particularly cycloheximide), and agar-agar when the medium is intended for use in a solid form.

In an embodiment, in the culture medium according to the invention the nutrient base is "Yeast Nitrogen Base" and the acid-base indicator is bromocresol green.

In another embodiment, the culture medium according to the invention further contains a bacterial growth inhibitor, particularly chloramphenicol and/or oxytetracycline, which is specially useful for the detection of yeasts of the *Dekkera* and *Brettanomyces* genera within mixed bacteria including

populations.

The medium object of the invention, when in the liquid form, is prepared by sterilizing filtration, then being dispensed into adequate test tubes. When the medium is desired in the solid form, agar-agar is dissolved in demineralized water and then sterilized in an autoclave; following the sterilization and cooling to about 50°C, the other components are added under aseptic conditions, having previously been filtration sterilized. The pH adjustment for both media is done before the sterilization, to a pH value that depends on the acid-base indicator used. The mixture is homogenized and poured into Petri dishes, the medium being ready for use after solidifying.

The present invention also refers to a method for the detection and enumeration of yeasts of the *Dekkera* and *Brettanomyces* genera, using a partially selective and totally differential culture medium, characterized as above.

The method according to the invention allows the detection and enumeration of yeasts of the *Dekkera* and *Brettanomyces* genera in a simple way using the culture medium according to the present invention.

According to the invention, the culture medium is used by direct contact with a sample under analysis, adequate dilutions being used if the enumeration of the contaminant yeasts is intended. The thus inoculated medium is then incubated at an adequate temperature for the growth of the yeasts, during a time period enough, usually 5 to 10 days, for the development of clearly visible colonies (in case of the solid medium) or for the clouding of the solution (in case of the liquid medium). The detection of the yeasts of the *Dekkera* and *Brettanomyces* genera in the Petri dishes is accomplished by direct observation of cream colored colonies, by the change of medium color according to the type of acid-base indicator that was used, and by the presence of a

characteristic phenol-like aroma. If the incubation time is extended, the colonies acquire a darker coloring. The detection in the liquid medium is accomplished by the change of the medium color and by the presence of the phenol-like aroma.

In addition, it is a convenient, swift, and easily reproducible procedure by any microbiological culture media manufacturing laboratory, without the need of the use of new technologies. Once the culture medium is manufactured, its use by any food and beverage industry or quality control laboratory is immediate, since it does not require specialized operators other than the ones in charge of routine microbiological analyses.

Therefore, one of the objectives of the present invention consists in providing the food industry with a procedure that allows the isolation, differential detection and enumeration of yeasts of the *Dekkera* and *Brettanomyces* genera by the production of characteristic colonies, medium color change and production of a phenol-like aroma, thus avoiding the inconveniences of said yeasts identification after their isolation, object being accomplished using the culture medium and method according to the present invention, as described above.

Thus, the use of the medium according to the invention allows:

a) the detection and identification of contaminations in the food and beverage industry due to yeasts of the *Dekkera* and *Brettanomyces* genera, in every step of the manufacturing process, from the raw materials to the finished and stored product.

b) the definition of the critical control points, in order to establish control criteria suited to each one of these points in the food and beverage industry.

Further, the medium according to the invention is useful for inclusion in yeast identification galleries.

Preferred Embodiments of the Invention

~~In a preferred embodiment, the present invention refers to a culture medium for the differential detection and enumeration of yeasts of the *Dekkera* and *Brettanomyces* genera which comprises "Yeast Nitrogen Base" as a nutrient base, ethanol as a non-fermentable energy source, *p*-cumaric acid as an aromatic compound (4-ethylphenol) promoting substrate, bromocresol green as an acid-base indicator with turning points in the acid range, an antibiotic that inhibits several species of yeasts (cycloheximide), and a bacterial growth inhibitor antibiotic.~~

Insert page 6a

In this embodiment of the invention, after culture medium inoculation with a sample containing yeasts of the *Dekkera* and *Brettanomyces* genera, which may be a previously isolated sample of these yeasts, or a mixed sample of yeasts and/or yeasts and bacteria, and incubation under advantageous growth conditions for these yeasts genera, after about 5 to 12 days, identification is possible by a culture medium color change, from blue to yellow, development of cream colored colonies and the characteristic phenol-like aroma.

The present invention is further illustrated by means of the following examples, which are intended only to exemplify and by no means limit the scope of the invention.

Examples

Example 1:

Preparation of a culture medium according to the invention

The culture medium, object of the present invention, can be prepared using

In a preferred embodiment, the present invention refers to a culture medium for the differential detection and enumeration of yeasts of the *Dekkera* and *Brettanomyces* genera which comprises "Yeast Nitrogen Base" as a nutrient base in an amount from 5 to 10 g/L, preferably 6.7 g/L, ethanol as a non-fermentable energy source in an amount from 32 to 96 g/L, preferably 48 g/L, *p*-cumaric acid as an aromatic compound (4-ethylphenol) promoting substrate, in an amount from 0.05 to 1.0 g/L, preferably 0.1 g/L, bromocresol green as an acid-base indicator with turning points in the acid range, an antibiotic that inhibits several species of yeasts (cycloheximide) in an amount from 0.004 to 0.1 g/L, preferably 0,01 g/L, and a bacterial growth inhibitor antibiotic.

the following formulation (g/L): Yeast Nitrogen Base (6.7), as the nutrient base; ethanol (48), as the non-fermentable energy source and as an inhibitor for some of the yeasts; *p*-cumaric acid (0.1), as the phenol-like producing aroma substract; bromocresol green (~~0.222~~ 0.022), previously dissolved in NaOH, as the acid-base indicator; cycloheximide (0.01), as the inhibitor antibiotic for some of the yeast species; chloramphenicol (0.1) and/or oxytetracycline (0.1), as the bacteria inhibitor antibiotic; and agar-agar (20), as the gelling agent. The culture medium is sterilized according to the following: the agar-agar is dissolved in 70% of the total needed water, the pH is adjusted to 5.4 with a strong acid, and the resulting solution is sterilized in an autoclave at 120°C, for 20 minutes. The other components are dissolved in the remaining of the demineralized water, the pH is adjusted to 5.4 with a strong acid, and the resulting solution is sterilized by filtration through a 0.22 μm pore diameter membrane. Both of the above solutions are then mixed together when the agar-agar solution reaches 50°C. The medium is then homogenized and dispensed into Petri dishes, allowing it to solidify prior to the inoculation.

Example 2:

Use of the culture medium object of the invention for the detection of yeasts of the* Dekkera *and* Brettanomyces *genera in wines

In this example, two wines suspected of having been altered were analyzed using the culture medium of Example 1. 20 ml samples of each wine were filtered under aseptic conditions, through 0,22 μm pore diameter cellulose acetate membranes. Each membrane was placed on the surface of a Petri dish containing the medium of the invention and incubated at 25°C. After 3 days it was possible to observe colonies in one of the dishes, along with the change of the medium color from blue to yellow; when the dish was opened, the presence of a phenol-like aroma was not detected. In the other dish no colonies were detect after 3 days. After 9 days the dish where the colonies had been observed maintained the same characteristics. In the other dish it was possible to observe small cream colored colonies, a color change of the

medium from blue to yellow and a characteristic phenol-like aroma. Using the classical identification methods it was confirmed that the colonies developed in the dish that did not show a phenol-like aroma did not belong to the *Dekkera* or *Brettanomyces* genera, while those of the dish that showed the phenol-like aroma belonged to these genera.

Example 3

Use of the culture medium object of the invention for the enumeration of yeasts in wines where the presence of filamentous fungi is suspected

In this case, when the development of molds on the surface of the Petri dishes can shield or inhibit the outcome of yeasts colonies, the Most Probable Number enumeration technique is used, using test tubes with the liquid culture medium. Consequently, agar-agar is not used in the medium formulation, the medium being completely sterilized by filtration. In this instance, the presence of yeasts of the *Dekkera* or *Brettanomyces* genera is detected when there is a clouding of the medium, a turning of its color from blue to yellow, and the presence of a phenol-like aroma; therefore, all the test tubes that show these characteristics are considered positive, and all the others negative.

Although the present invention is described based on its preferred embodiments, it should be apparent to any person skilled in the art that variations and modifications within the spirit and scope of the appended claims are possible.

CLAIMS

1 – A differential culture medium for the enumeration of food and beverage contaminant yeasts of the *Dekkera* and *Brettanomyces* genera, characterized in that it comprises a nutrient base, ethanol, *p*-cumaric acid, an acid-base sensitive indicator with turning points in the acid range, an inhibitor antibiotic for ~~some~~ yeasts species, and optionally a bacteria growth inhibitor and agar-agar.

2 – A culture medium according to claim 1, characterized in that the present amount of ethanol is from 32 to 96 g/L, preferably 48 g/L.

3 – A culture medium according to claim 1, characterized in that the present amount of *p*-cumaric acid is from 0.05 to 1.0 g/L, preferably 0.1 g/L.

4 – A culture medium according to claim 1, characterized in that the nutrient base is "Yeast Nitrogen Base", in amounts from 5 to 10 g/L, preferably 6.7 g/L.

5 – A culture medium according to claim 1, characterized in that the inhibitor antibiotic for some of the yeast species is cycloheximide, present in an amount from 0.004 to 0.1 g/L, preferably 0.01 g/L.

6 – A culture medium according to claim 1, characterized in that the pH indicator is bromocresol green, present in an amount of ~~about~~ 0.022 g/L.

7 – A culture medium according to claim 6, characterized in that the medium pH is adjusted with a strong acid to a value between 4.8 and 6.0, preferably 5.4.

8 – A culture medium according to claim 1, characterized in that it additionally contains a bacteria growth inhibitor, preferably chloramphenicol and/or

oxytetracycline, in amounts of ~~about~~ 0.1 g/L, to detect and identify yeasts of the *Dekkera* and *Brettanomyces* genera in food and beverage products containing mixed populations of yeasts and bacteria.

9 – A culture medium according to any one of the preceding claims, characterized in that it contains all the components except agar-agar, to detect and identify yeasts of the *Dekkera* and *Brettanomyces* genera in food and beverage products containing mixed populations of yeasts, bacteria and particularly filamentous fungi.

10 – A differential culture medium for enumeration of food and beverage contaminant yeasts of the *Dekkera* and *Brettanomyces* genera, characterized in that it has the following composition: 5 to 10 g/L, preferably 6.7 g/L, of "Yeast Nitrogen Base"; 0.004 to 0.1 g/L, preferably 0.01 g/L, of cycloheximide; 0.05 to 1.0 g/L, preferably 0.1 g/L, of *p*-cumaric acid; 0.022 g/L of bromocresol green, or another acid-base indicator with similar turning points; 32 to 96 g/L, preferably 48 g/L, of ethanol; 0.1 g/L of chloramphenicol and/or 0.1 g/L of oxytetracycline, and 20 g/L of agar-agar, the pH of the medium being adjusted between 4.8 and 6.0, preferably 5.4, with a strong acid.

11– A differential culture medium for enumeration of food and beverage contaminant yeasts of the *Dekkera* and *Brettanomyces* genera according to the preceding claims, characterized by the sterilization of all the components is done by filtration, except for the agar-agar which is sterilized in autoclave; the addition under aseptic conditions to this solution, after agar-agar cooling and before it solidifies, of all the other components of the medium, previously sterilized by filtration; and the dispensing of the medium into Petri dishes so that it solidifies.

12 – A process for the detection and/or identification of yeasts of the *Dekkera*

and *Brettanomyces* genera, characterized by the use of a differential culture medium for enumeration of food and beverage contaminant yeasts of the *Dekkera* and *Brettanomyces* genera, comprising a nutrient base, a non-fermentable energy source, *p*-cumaric acid, an acid-base indicator with turning points in the acid range, an inhibitor antibiotic for some of the yeast species, and optionally a bacteria growth inhibitor and agar-agar.

13 – A process for the detection and/or identification of yeasts of the *Dekkera* and *Brettanomyces* genera according to claim 12, characterized in that the acid-base indicator is bromocresol green, and after inoculation of said medium with a sample containing yeasts of the *Dekkera* and *Brettanomyces* genera, and incubation for 5 to 12 days in adequate conditions for the growth of said yeasts, it is possible to detect the presence, and if needed the enumeration of said yeasts genera, by means of a medium color change, from blue to yellow, and development of cream colored colonies and a phenol-like aroma, characteristic of the yeasts of the *Dekkera* and *Brettanomyces* genera.

14 – A process for the detection and/or identification of yeasts of the *Dekkera* and *Brettanomyces* genera according to claims 12 and 13, characterized in that it is applied to the detection and enumeration of yeasts of the *Dekkera* and *Brettanomyces* genera in food and beverage products samples.

15 – Use of a culture medium according to claims 1 to 11, for inclusion in an identification gallery, together with other yeast identification tests.

16 – Use of a culture medium according to claims 1 to 11 in an industry, particularly in the quality and process control in a food and beverage industry.

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(54) Title: CULTURE MEDIUM FOR DETECTION OF *DEKKERA* AND *BRETTANOMYCES*

(57) Abstract: The present invention provides a generic culture medium for the detection and enumeration of yeasts belonging to the *Dekkera* and *Brettanomyces* genera and a method for the detection and enumeration of said yeasts using said culture medium. According to the invention, the method comprises adding to a base yeast culture medium, a non fermentable energy source, particularly ethanol, *p*-cumaric acid, as an aromatic compound promoting substract, exclusively produced by said yeast genera, an acid-base indicator, particularly bromocresol green, a yeast growth inhibitor antibiotic, particularly cycloheximide, and a bacterial growth inhibiting antibiotic, particularly chloramphenicol and/or oxytetracycline. When yeasts of the genera *Dekkera* and *Brettanomyces* are cultivated in said medium, the developed colonies show a characteristic color, the culture medium color changes according a reproducible pattern, due to the decrease in pH, and a characteristic phenol-like aroma is developed, easily detectable by smell after a few days of incubation, which allows their detection and enumeration. The invention is useful in the detection and enumeration of yeasts belonging to the *Dekkera* and *Brettanomyces* genera in the food and beverage industry, allowing its inclusion in yeast identification galleries.

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NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

FERREIRA MAGNO, Fernando António
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PORTUGAL

Date of mailing (day/month/year)
15 November 2001 (15.11.01)

Applicant's or agent's file reference
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IMPORTANT NOTIFICATION

International application No.
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1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

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2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

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State of Residence: PT
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3. Further observations, if necessary:

4. A copy of this notification has been sent to:

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Declaration, Power of Attorney and Petition

WE (I) the undersigned inventor(s), hereby declare(s) that:

My residence, post office address and citizenship are as stated below next to my name,

We (I) believe that we are (I am) the original, first and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

CULTURE MEDIUM FOR DETECTION OF DEKKERA AND BRETTANOMYCES

the specification of which

☐ is attached hereto.

☐ was filed on _____ as

Application Serial No. _____

and amended on _____.

☒ was filed as PCT international application

Number PCT/PT00/00005

on May 31, 2000,

and was amended under PCT Article 19

on _____ (if applicable).

We (I) hereby state that we (I) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We (I) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

We (I) hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed. Prior Foreign Application(s)

Application No.	Country	Day/Month/Year	Priority Claimed
102306	Portugal	31 May 1999	☒ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No

We (I) hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

(Application Number) (Filing Date)

(Application Number) (Filing Date)

We (I) hereby claim the benefit under 35 U S.C § 120 of any United States application(s), or under § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

Application Serial No.	Filing Date	Status (pending, patented, abandoned)
PCT/PT00/00005	31 May 2000	

And we (I) hereby appoint the following registered practitioner(s):

22850

as our (my) attorneys, with full powers of substitution and revocation, to prosecute this application and to transact all business in the Patent Office connected therewith; and we (I) hereby request that all correspondence regarding this application be sent to

22850

We (I) declare that all statements made herein of our (my) own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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